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The role of dorsomedial hypotalamus ionotropic glutamate receptors in the hypertensive and tachycardic responses evoked by Tityustoxin intracerebroventricular injection

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ABSTRACT

The scorpion envenoming syndrome is an important worldwide public health problem due to its high incidence and potential severity of symptoms. Some studies address the high sensitivity of the central nervous system to this toxin action. It is known that cardiorespiratory manifestations involve the activation of the autonomic nervous system. However, the origin of this modulation remains unclear. Considering the important participation of the dorsomedial hypotalamus (DMH) in the cardiovascular responses during emergencial situations, the aim of this work is to investigate the involvement of the DMH on cardiovascular responses induced by intracerebroventricular (icv) injection of Tityustoxin (TsTX, a α-type toxin extracted from the *Tityus serrulatus* scorpion venom). Urethane-anaesthetized male Wistar rats ($n = 30$) were treated with PBS, muscimol or ionotropic glutamate receptor antagonists, bilaterally in DMH and later, with an icv injection of TsTX, or treated only with PBS in both regions. TsTX evoked a marked increase in mean arterial pressure and heart rate in all control rats. Interestingly, injection of muscimol, a $GABA_A$ receptor agonist, did not change the pressor and tachycardic responses evoked by TsTX. Remarkably, the injection ionotropic glutamate receptors antagonists in DMH abolished the pressor and the tachycardic response evoked by TsTX. Our data suggest that the central circuit recruited by TsTX, whose activation results in an array of physiological and behavioral alterations, depend on the activation of DMH ionotropic glutamate receptors. Moreover, our data provide new insights on the central mechanisms involved in the development of symptoms in the severe scorpion envenomation syndrome.

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1. Introduction

The severe scorpion envenomation syndrome (SSE) is a medical-sanitary condition with high death incidence in infants (Chippaux, 2012; Chippaux and Goyffon, 2008; Fundação Nacional de Saúde, 2001; Ministério da Saúde, 2009). *Tityus serrulatus*, the scorpion of larger medical relevance in Brazil, is responsible for the most severe accidents (Fundação Nacional de Saúde, 2001). Its venom is composed by a complex mixture of toxic and non-toxic peptides (Diniz and Gonçalves, 1960). Among many toxic substances, tityustoxin (TsTX) is considered the one of the most lethal component (Kalapothakis and Chavez-Olortegui, 1997) of the venom. TsTX, an α-type toxin, binds to site 3 of voltage-gated sodium channels (VGSC), mainly during the activated state. It delays VGSC inactivation and increases the permeability to sodium in the cell membrane, which, consequently, enhances neurotransmitters release, triggering several systemic disorders (Barhanin et al., 1982; Casali et al., 1995; Dorce and Sandoval, 1994; Massensini et al., 1998).

Along the last decades, many studies pointed out the cardiorespiratory complications (cardiac arrhythmias, sustained arterial hypertension and terminal hypotension, pulmonary edema and circulatory failure) as the main "*causa mortis*" of scorpion envenoming (Bahloul et al., 2002; Freire-Maia and Campos, 1989; Freire-Maia et al., 1994, 1974; Ismail, 1995). There is an overwhelming experimental literature about the effects of *T. serrulatus* scorpion venom and its neurotoxins on cardiovascular system (Azevedo et al., 1983; Celeste Henriques et al., 1968; Efrati, 1951; Freire-Maia et al., 1974; Guidine et al., 2009; Ismail, 1995; Ismail et al., 1972, 1973, 1974; Magalhaes, 1928; Mesquita et al., 2003; Mohammed, 1942). Regardless of the administration route and/or the animal's conscious state, the most related symptoms are blood pressure changes, cardiac arrhythmias and pulmonary edema (Ismail, 1995). Nevertheless, the precise venom and toxin mechanisms of action are still divergent, several mechanisms have been proposed (e.g., peripheral sympathetic stimulating effect; central spinal and sympathetic preganglionic stimulation consequence; adrenal medullary secretory outcome; direct action on heart; medullary or hypothalamic stimulation result; and a combination of all or some of those effects) (Celeste Henriques et al., 1968; Del Pozo, 1968; Efrati, 1951; Freire-Maia et al., 1974; Guidine et al., 2009; Ismail, 1995; Magalhaes, 1928; Mesquita et al., 2002; Teixeira et al., 2001). Nonetheless, there is a consensus, among the investigators, that the cardiovascular effects of scorpion toxins are mediated by the activation of the autonomic nervous system (ANS), prominently by the sympathetic branch and the release of tissue and medullary catecholamines (Freire-Maia et al., 1974; Ismail, 1994, 1995; Ito et al., 1981). However, the origin of this modulation remains unclear.

There is strong evidence that central nervous system (CNS) plays a key role in the genesis of SSE symptoms. Intracerebroventricular (icv) injections of a low dose of TsTX (1.74 μg) in adult rats induced typical symptoms of severe scorpion envenoming such as: tremors, piloerection, tachypnea, convulsions, cardiac arrhythmias, tachycardia, hypertension and death (Mesquita et al., 2003; Silva et al., 2013). Meanwhile, iv administration of this dose failed to produce the aforementioned effects (Mesquita et al., 2003). Moreover, a subcutaneous (sc) injection of a higher dose of TsTX (6 mg/kg) in weanling rats induced high amplitude discharges in the nucleus tractus solitarius (NTS), which were correlated to electrocardiographic changes (e.g., atrioventricular blocks of different degrees, ectopic beats, sinus tachycardia or bradycardia and premature atrial and ventricular depolarization) (Guidine et al., 2009). In addition, a recent experimental study showed that brainstem areas involved in neurovegetative regulation were most likely within the primary structures triggering the cascade of symptoms present in SSE (Guidine et al., 2014).

Furthermore, encephalic structures, crucial to cardiovascular control, such as dorsomedial hypothalamus (DMH), could be susceptible to the action of the substances in circulation (Guyenet, 2006; Price et al., 2008). In fact, the DMH is a key component of the central pathways mediating the cardiovascular responses during defensive reactions (DiMicco et al., 1996; Graeff, 1990; Jardim and Guimaraes, 2001). Although the DMH is constantly under the inhibition of the medial pre-optic area, it may receive excitatory inputs from other regions, such as: amygdala (Amg) and periaquedutal gray (PAG), which also contribute to cardiovascular and behavioral defense responses (de Menezes et al., 2006, 2008, 2009; Horiuchi et al., 2009; Soltis et al., 1998). The DMH also sends projections to the rostroventrolateral medulla (RVLM) and raphe palidus (RP) (Fontes et al., 2001, 2006; Horiuchi et al., 2004), which have an important regulatory role over vasomotor and cardiac sympathetic tonus (Fontes et al., 2001, 2006, 2011). Moreover, studies have shown that the injections of GABA receptor antagonists and excitatory amino acids into DMH elicited marked increases in heart rate and blood pressure in anesthetized and conscious rats, showing the important participation of the glutamatergic and GABAergic neurotransmission systems in the integration and control of the cardiovascular parameters during the defense reaction (DiMicco and Abshire, 1987; DiMicco et al., 1986, 2002). All the observations above indicate that the DMH neurons may be critical targets in the cardiovascular response elicited by icv injection of TsTX, suggesting that this nucleus activation could have important role in the pathogenesis of the cardiovascular complications elicited by central action of such toxin. Thus, the aim of this work is to investigate the involvement of glutamatergic and GABAergic neurotransmission within the DMH on these cardiovascular changes induced by the icv injection of TsTX. For that, we evaluated the effect of acute chemical inhibition of the DMH neurons, by injecting muscimol (a $GABA_A$ receptor agonist), on the cardiovascular responses evoked by the icv injection of TsTX. We also evaluated the role of DMH ionotropic glutamate receptors in these responses by injecting 2-amino-5-phosphonopentanoate (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione) (CNQX), antagonists of NMDA and AMPA/kainate receptor subtypes, respectively, into the DMH.

2. Materials and methods

2.1. Scorpion toxin and drugs

Tityustoxin (TsTX) was isolated from the venom of *T. serrulatus* scorpion as described by Gomez and Diniz (1966) and modified by Sampaio et al. (1983). The lyophilized toxin was solubilized in 500 μL of phosphate buffered saline (PBS). A known concentration of TsTX, as determined by Hartree (1972), had serum bovine albumin as standard, was used to determine the absorbance coefficient read at 280 nm: [protein](Ag/ml)/A280 = 279. Further determination of TsTX concentration was done by the direct reading of samples in the spectrophotometer (Hitachi spectrophotometer, model 2001, Japan). After determining the protein concentration (4.76 μg/μL), the initial pool was stored in volumes of 10 μL each, and stored at −20 °C until the time of the experiments. All experiments used the same initial pool of TsTX. Muscimol, AP5 and CNQX were purchased from Sigma, USA.

2.2. Animals

Experiments were performed on male Wistar rats ($n = 30$; 300 ± 10 g), supplied by the Center of Animal Science/UFOP. They were kept in grouped cages ($n = 3$) on a 12 h light/dark cycle, at a controlled room temperature (23 °C), and fed with commercial chow

and filtered water ad libitum. Efforts were made to avoid any unnecessary distress to the rats, in accordance to the Brazilian Council for Animal Experimentation. All procedures were approved by the institutional ethics committee for animal research of the Federal University of Ouro Preto (CEUA-UFOP; 2011/46; 2013/49), and were performed according to the regulations set forth by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.3. Surgical procedures

Experiments were conducted under urethane (1.2–1.4 g/kg, ip) anesthesia. Its adequacy was verified by the absence of a withdrawal response to a nociceptive stimulation of a hindpaw. Supplemental doses of urethane iv were given when necessary. Following anesthesia induction, an endotracheal tube was inserted in order to facilitate the aspiration and the airway maintenance. Polyethylene catheters were placed into the femoral artery and vein for arterial pressure (AP) recordings and drug injections, respectively. Subsequently, the animals were positioned on a heating pad in a prone position, and their heads were placed in a stereotaxic frame (Stoelting, Wood Dale, IL), with the tooth bar fixed at −3.3 mm below the interaural line. A small craniotomy was made bilaterally near the bregma level to allow later insertion of an ultrafine injector into the left lateral ventricle (LLV) and DMH (stereotaxic coordinates for LLV: AP −0.5; LL −1.5; DV −4.7; and for DMH: AP −3.3; LL ±0.6; DV −8.3) (Paxinos and Watson, 1986). Body temperature was monitored using rectal thermometer and maintained in the range of 37–37.5 °C using a heating pad.

2.4. Experimental design

All experiments started only after stabilization of physiological parameters (MAP, HR and temperature) for at least 30 min. The microinjections were performed by an ultrafine injector needle (dental needle, G30, 11 mm of length) connected to 5 μL Hamilton by a polyethylene tube (PE-10 Intramedic, Clay Adams) filled with distilled water. The microinjection was considered successful if the bubble air, previously done, move in polyethylene tube, indicating that the injector was not obstructed. The microinjections in DMH and LLV were done, respectively, during 10 and 30 s.

Experiment 1. Effects of a low dose of TsTX icv injection on mean arterial pressure (MAP) and heart rate (HR) ($n = 5$).

The first experiment was performed in order to test the influence of a low dose of TsTX icv injection on MAP and HR. For this end, the animals were submitted to basal period record during 20 min, followed by PBS injections (100 nL) into the right and left side of the DMH, randomly chosen. After 12 min, they were submitted to TsTX injection (0.116 μg; 1 μL) into LLV. The cardiovascular changes evoked by TsTX were recorded for 60 min. This TsTX dose was chosen based on earlier studies demonstrating that icv injection of higher dose of TsTX (1.74 μg) in adult rats induced typical symptoms of severe scorpion envenoming such as: tachypnea, convulsions, cardiac arrhythmias, tachycardia, hypertension and death (Mesquita et al., 2003; Silva et al., 2013). Considering this, we performed a curve dose-effect with different doses (1.74 μg; 0.174 μg; 0.116 μg and 0.087 μg). The lower dose tested which could reproduce some of the cardiovascular symptoms aforementioned, without cause early death in rats was 0.116 μg (unpublished data). For comparison, the same aforementioned parameters will be evaluated at another group ($n = 4$) submitted to PBS injection only, both in the DMH and in the LLV.

Experiment 2. Effects of DMH inhibition by a GABAergic receptors agonist on the cardiovascular response evoked by TsTX icv injection ($n = 5$).

The second experiment was done in order to determine the effect of the activation of DMH GABAergic receptors on cardiovascular responses evoked by TsTX icv injection. For this end, the animals were submitted to basal period record during 20 min, followed by an injection of muscimol ($GABA_A$ receptor agonist; 100 pmol; 100 nL) into the right and left side of the DMH, randomly chosen. After 12 min, they were submitted to TsTX injection (0.116 μg; 1 μL) into LLV. The cardiovascular changes evoked by TsTX were also recorded for 60 min. This dose of muscimol was chosen based on earlier studies demonstrating that microinjecting this same dose in the DMH effectively reduced the cardiovascular responses evoked by acute stress (de Menezes et al., 2009).

Experiment 3. Effects of DMH inhibition by glutamate ionotropic receptors antagonists on the cardiovascular response evoked by TsTX icv injection ($n = 16$).

The third experiment was done in order to verify the contribution of DMH ionotropic glutamatergic receptors on cardiovascular responses mediated by TsTX icv injection. Thus, the animals were submitted to basal period record during 20 min, followed by an injections of: (a) a mixture of glutamate ionotropic receptor antagonists (AP5 and CNQX, 100 pmol/100 nL; n = 5), b) AP5 (200 pmol/100 nL; $n = 6$) or c) CNQX (200 pmol/100 nL; $n = 5$) into the right and left side of the DMH, randomly chosen. After 12 min, they were submitted to TsTX injection (0.116 μg; 1 μL) into LLV. The cardiovascular changes evoked by TsTX were also recorded for 60 min. This dose of the ionotropic glutamate receptors antagonists mixture was chosen based on earlier studies demonstrating that microinjection of the same dose in the DMH effectively reduced the cardiovascular responses evoked by acute stress (de Menezes et al., 2009; Soltis and DiMicco, 1992). Additionally, the chosen doses of these antagonists injected separately corresponded to double of the mixture chosen dose, since it was crucial to promote the total blockade of tachycardia mediated by TsTX icv injection.

2.5. Histology

After the recordings, animals were euthanized and Evans blue dye (1 μL; icv route) was injected in order to confirm the injection sites. The brains were removed and kept in: 10% formaldehyde for at least 48 h and in 20% sucrose for additional 24 h. Then, they were sliced in a cryostat (50 μm thickness). The slices were mounted on glass slides. After drying, the slides were stained with neutral red and visualized in an optical microscope for confirmation of DMH and LLV injections sites (Supplementary Fig. S1). Rats without confirmed histology were discarded from the study.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.12.006.

2.6. Data analysis

For statistical analysis and for representation in table and figures, the baseline MAP and HR values were calculated by the mean of the values obtained during 2 min prior to the first injection (Basal period). The average changes from baseline were calculated during 6 min after the PBS, muscimol or any glutamate receptor antagonists injection (PBS, muscimol or glutamate antagonists period) and throughout period after TsTX injection (TsTX period).

Table 1
Average baseline values for mean arterial pressure (MAP) and heart rate (HR), regarding the experimental series.

	DMH/LLV PBS ($n=4$)	DMH PBS ($n=5$)	DMH muscimol ($n=5$)	DMH mixture ($n=5$)	DMH AP5 ($n=6$)	DMH CNQX ($n=5$)
MAP baseline (mmHg)	83 ± 8	78 ± 3	81 ± 6	82 ± 6	95 ± 6	83 ± 5
HR baseline (bpm)	346 ± 17	383 ± 8	384 ± 21	381 ± 17	384 ± 12	388 ± 9

2.7. Statistical analysis

Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze all data. Data were expressed as mean ± standard error of mean (mean ± SEM). One-way ANOVA was used to compare the basal period of all groups and to compare the total area under the curve of mean arterial pressure and heart rate, followed by Dunnett's post hoc. Two-way ANOVA was used to analyze all groups considering the influence of time and treatment, and was followed by Bonferroni post hoc. The significance level was fixed at 5%.

3. Results

Baseline MAP and HR were not different between groups (Table 1). In order to confirm that central nervous system (CNS) plays an important role in the genesis of SSE symptoms we injected a low dose of TsTX (0.116 μg/μL; icv). Intracerebroventricular injection of PBS did not change MAP and HR in animals that also received a PBS injection into the DMH. Intracerebroventricular injection of the aforementioned dose of TsTX evoked high pressor and tachycardic responses in all animals, which also received an injection of PBS in the DMH (for treatment DMH/VLE PBS vs. DMH PBS; ΔMAP: $F_{(1,\ 264)} = 371$, $p < 0.0001$; ΔFC: $F_{(1,\ 231)} = 401$, $p < 0.0001$; Fig. 1). In order to evaluate the involviment of DMH neurons on the physiological responses induced by TsTX, first we investigated the influence of its GABAergic receptors on such responses. Thus, we injected muscimol (a $GABA_A$ receptor agonist) (100 pmol/100 nL) into the DMH. Interestingly, the DMH chemical inhibition did not change the pressor and tachycardic responses evoked by TsTX (for treatment DMH PBS vs. DMH muscimol; ΔMAP: $F_{(1,\ 264)} = 0.03$, $p > 0.05$; ΔFC: $F_{(1,\ 264)} = 3.06$, $p > 0.05$; Fig. 2). Moreover, we investigated the role of the glutamatergic receptor within the DMH on the physiological responses produced by the icv injetion of TsTX. Remarkably, the injection of a mixture of AP5 and CNQX in DMH abolished the pressor and decreased the tachycardic response evoked by TsTX (for treatment DMH PBS vs. DMH mixture; ΔMAP: $F_{(1,\ 264)} = 175.7$, $p < 0.0001$; ΔFC: $F_{(1,\ 264)} = 105.1$, $p < 0.0001$; Fig. 3). Additionally, the injection of AP5 alone in DMH abolished the pressor and tachycardic response evoked by TsTX (for treatment DMH PBS vs. DMH AP5; ΔMAP: $F_{(1,\ 297)} = 404.8$, $p < 0.0001$; ΔFC: $F_{(1,\ 264)} = 986.3$, $p < 0.0001$; Fig. 4). Likewise, the injection of CNQX alone in DMH also abolished the cardiovascular changes evoked by TsTX (for treatment DMH PBS vs. DMH CNQX; ΔMAP: $F_{(1,\ 264)} = 293.3$, $p < 0.0001$; ΔFC: $F_{(1,\ 264)} = 105.1$, $p < 0.0001$; Fig. 5). Additionaly, the analisys of the total area under the curve of MAP and HR along the time, showed an increase in DMH PBS and DMH muscimol when compared to DMH/LLV PBS (area under the curve of ΔMAP along the time: DMH/LLV PBS = 391 ± 131 [(mmHg) × (min)]; DMH PBS = 1179 ± 158 [(mmHg) × (min)] and DMH muscimol = 1272 ± 306 [(mmHg) × (min)], $p = 0.0063$; area under the curve of ΔHR along the time: DMH/LLV PBS = 1894 ± 274 [(bpm) × (min)]; DMH PBS = 5442 ± 548 [(bpm) × (min)] and DMH muscimol = 5259 ± 992 [(bpm) × (min)], $p = 0.0002$; Supplementary Fig. S2). The other groups did not show significative differences (area under the curve of ΔMAP along the time: DMH mixture = 387 ± 59 [(mmHg) × (min)]; DMH AP5 = 488 ± 145 [(mmHg) × (min)] and DMH CNQX = 563 ± 246 [(mmHg) × (min)], $p > 0.05$; Area under the curve of ΔHR along the time: DMH mixture = 3673 ± 646 [(bpm) × (min)]; DMH AP5 = 1216 ± 319 [(bpm) × (min)] and DMH CNQX = 1816 ± 935 [(bpm) × (min)], $p > 0.05$; Supplementary Fig. S2). Post-mortem histology confirmed that injection sites were located in the ventricle and DMH (Supplementary Fig. S1).

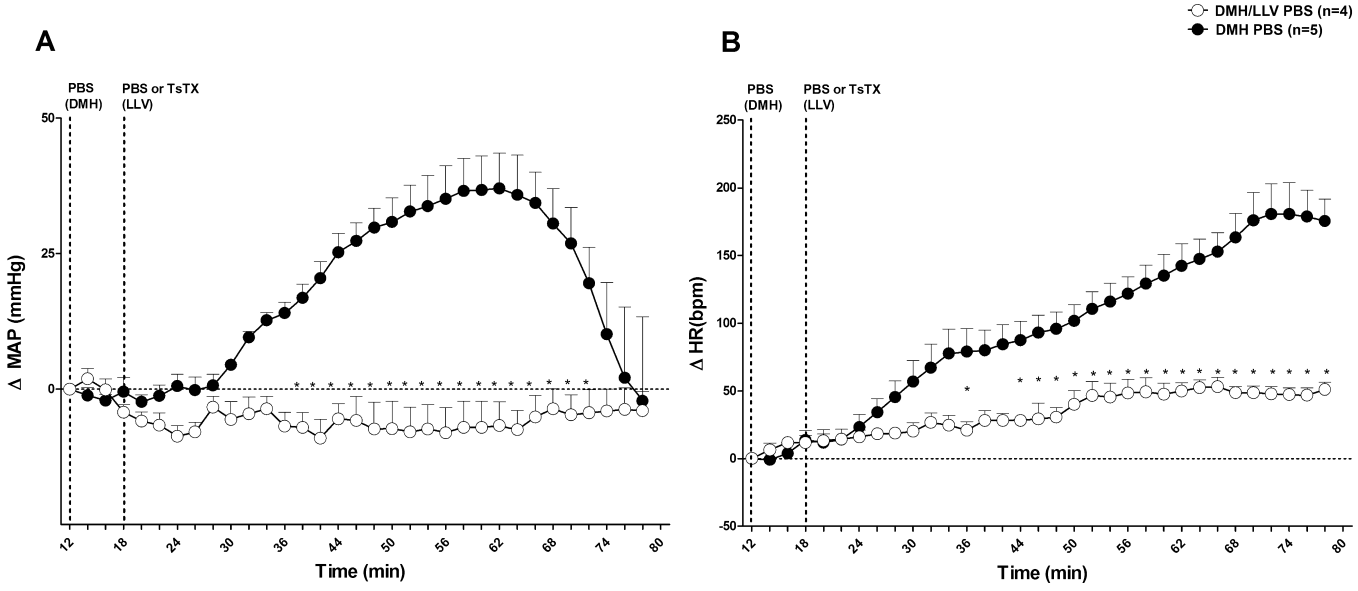

Fig. 1. Effect of a low dose of TsTX icv injection on mean arterial pressure and heart rate in Wistar rats anaesthetized with urethane. (A) Mean arterial pressure (MAP, mmHg) and (B) heart rate (HR, bpm) after PBS injection (100 nL) in DMH and PBS (1 μL) or TsTX injection (0.116 μg/1 μL) in left lateral ventricle (LLV). Symbols represent mean ± SEM. * $p<0.05$, DMH/LLV PBS vs DMH PBS (two-way ANOVA, Bonferroni post hoc). The dotted line illustrates the microinjections of PBS in DMH and PBS or TsTX in LLV.

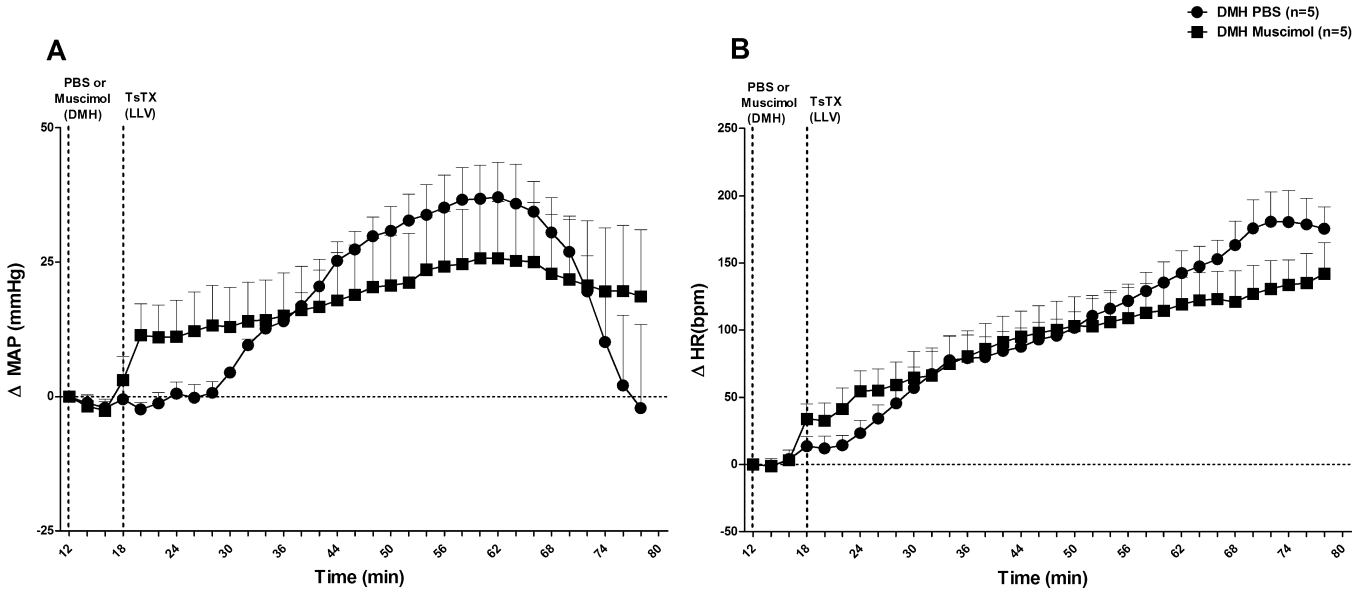

Fig. 2. Effect of DMH GABAergic receptor agonist on the cardiovascular responses evoked by TsTX injection in Wistar rats anaesthetized with urethane. (A) Mean arterial pressure (MAP, mmHg) and (B) heart rate (HR, bpm) after PBS (100 nL) or muscimol injection (100 pmol/100 nL) in DMH and TsTX injection (0.116 μg/1 μL) in left lateral ventricle (LLV). Symbols represent mean ± SEM. The dotted line illustrates the microinjections of PBS or muscimol in DMH and TsTX in LLV.

Supplementary Fig. S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.12.006.

4. Discussion

In this study, we evaluated the involvement of DMH neurons on the cardiovascular response induced by the central injection of TsTX. Our results showed that the hypertensive and tachycardic responses induced by TsTX depends on the activation of glutamate receptors present in the DMH. Our data provide new insights on the central mechanisms involved in the development of symptoms in the severe scorpion envenomation syndrome.

First, we assessed the effects of TsTX icv injections (0.116 μg/μL) on cardiovascular homeostasis of urethane-anaesthetized. We decided to use anaesthetized rats in order to abolish the behavioral effects of TsTX (e.g., tremors, convulsions, turns, jumps and runs), which usually generate artifacts compromising the analysis of cardiovascular parameters. TsTX icv injection induced intense pressor and tachycardic responses in all control animals, corroborating previous experiments using higher doses of TsTX in conscious rats (Mesquita et al., 2003; Silva et al., 2013). Additionally, other studies have also investigated peripheral consequences of the central injections of TsTX, pointing to a neurogenic nature of these responses. In this regard, TsTX icv injection, in Wistar rats, produced severe lung edema which was eliminated by an intramuscular injection of phenobarbital (a $GABA_A$ agonist) (Mesquita et al., 2002). Moreover, it also caused early cortical epileptiform discharges correlated temporally with cardiac arrhythmias, followed by death. All these symptoms were prevented by intraperitoneal carbamazepine injections (a sodium

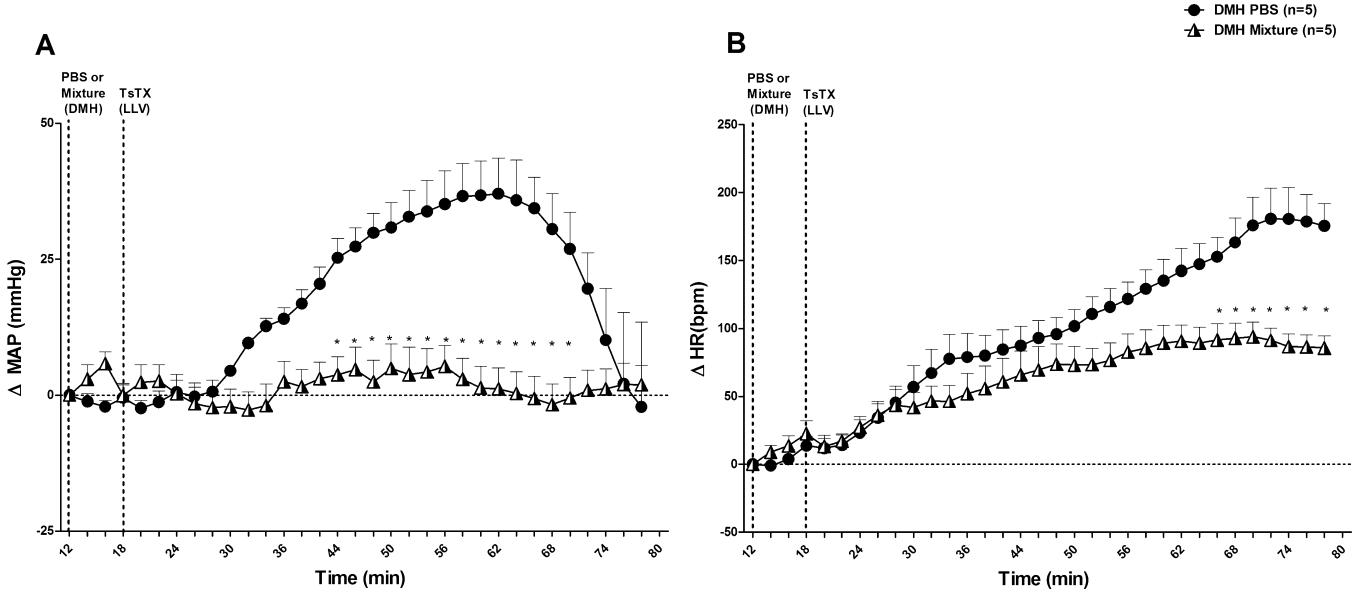

Fig. 3. Effect of the DMH glutamatergic inhibition on the cardiovascular responses evoked by TsTX injection in Wistar rats anaesthetized with urethane. (A) Mean arterial pressure (MAP, mmHg) and (B) heart rate (HR, bpm) after PBS (100 nL) or mixture injection (AP5 and CNQX, 100 pmol/100 nL) in DMH and TsTX injection (0.116 μg/1 μL) in LLV. Symbols represent mean ± SEM. $^*p < 0.05$, DMH PBS vs. DMH mixture (two-way ANOVA, Bonferroni post hoc). The dotted line illustrates the microinjections of PBS or mixture in DMH and TsTX in LLV.

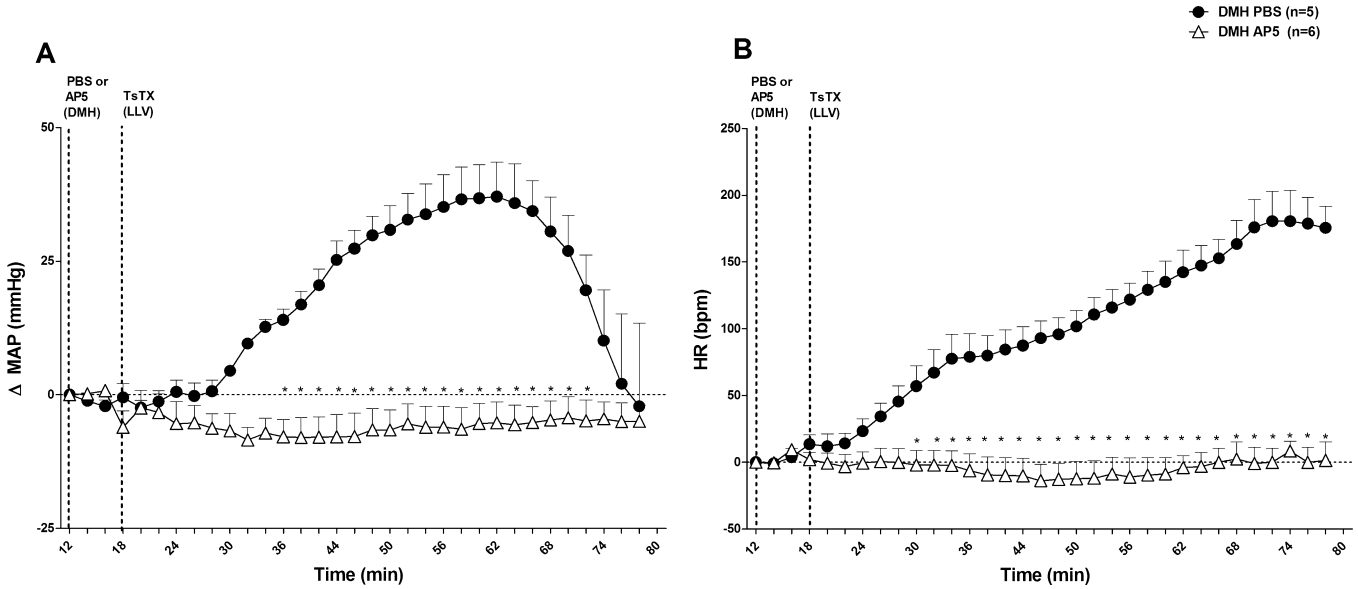

Fig. 4. The effect of the DMH NMDA receptor antagonist on the cardiovascular responses evoked by TsTX injection in Wistar rats anaesthetized with urethane. (A) Mean arterial pressure (MAP, mmHg) and (B) heart rate (HR, bpm) after PBS (100 nL) or AP5 injection (200 pmol/100 nL) in DMH and TsTX injection (0.116 μg/1 μL) in LLV. Symbols represent mean ± SEM. $^{*}p < 0.05$, DMH PBS vs. DMH AP5 (two-way ANOVA, Bonferroni post hoc). The dotted line illustrates the microinjections of PBS or AP5 in DMH and TsTX in LLV.

channels blocker) (Guidine et al., 2008b). In another study, a injection of a TsTX marked with a fluorescent agent, Alexa Fluor 568, produced an intense fluorescence signal in all periventricular areas, mainly in brainstem neurons (Guidine et al., 2014). Synthesis of the present results with the previous findings shows that that the CNS plays a pivotal role in the severity of scorpion intoxication.

Based in the results described above and in the fact that the activation of the DMH produces cardiovascular and behavioral responses (DiMicco et al., 1996; Graeff, 1990; Jardim and Guimaraes, 2001) similar to the ones induced by the icv injection of TsTX (Guidine et al., 2008b; Mesquita et al., 2002, 2003; Silva et al., 2013) we investigated the contribution of DMH neurons on cardiac and circulatory responses induced by the icv injection of TsTX. Thus, we investigated the effect of the DMH inhibition on the cardiovascular response evoked by TsTX (icv) injection using two protocols: (1) activating GABAergic receptors by injecting a $GABA_A$ agonist; and (2) blocking ionotropic glutamate receptors by injecting glutamatergic antagonists.

We initially investigated the effect of the activation DMH $GABA_A$ receptors on cardiovascular responses induced by TsTX. Microinjection of muscimol (a $GABA_A$ receptor agonist) in DMH did not change the profile of the pressor and tachycardic responses induced by TsTX injection in urethane-anaesthetized rats. Notably, TsTX increases VGSC depolarization time and, consequently, induces excessive neurotransmitters release, particularly of dopamine and glutamate (Fernandes et al., 2004; Massensini et al., 1998; Nencioni et al., 2003). This leads to neuronal hyperexcitability (in this case, TsTX-mediated) promoting an imbalance between brain excitation and inhibition, favoring

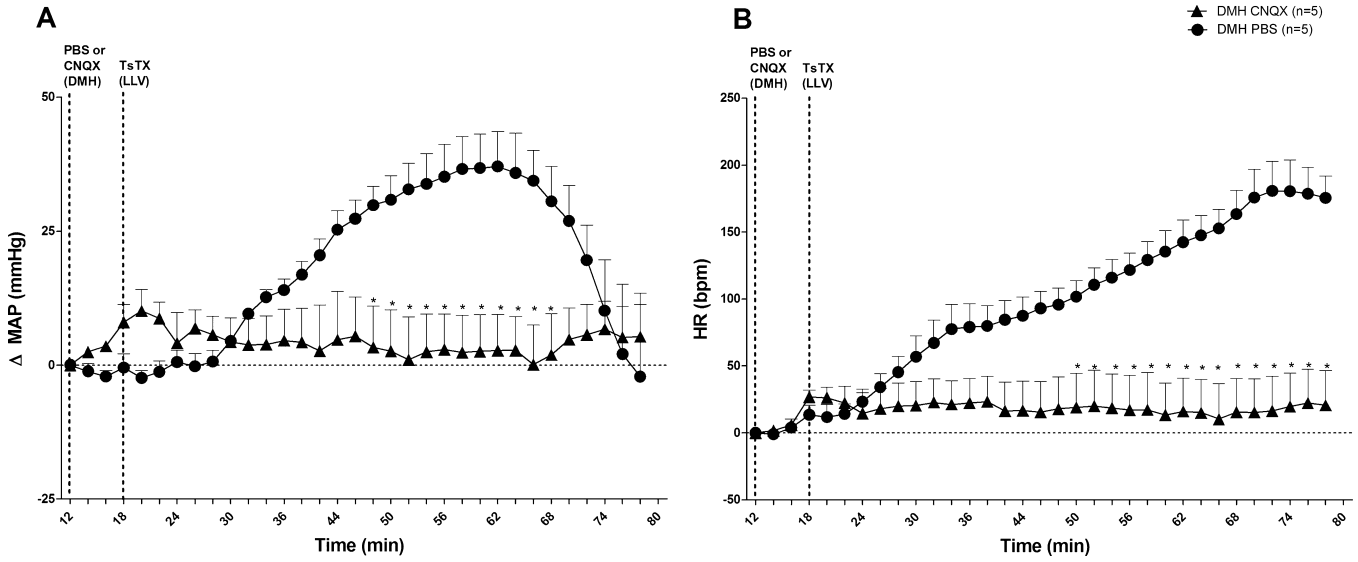

Fig. 5. The effect of the DMH AMPA/kainate receptor antagonist on the cardiovascular responses evoked by TsTX injection in Wistar rats anaesthetized with urethane. (A) Mean arterial pressure (MAP, mmHg) and (B) heart rate (HR, bpm) after PBS (100 nL)or CNQX injection (200 pmol/100 nL) in DMH and TsTX injection (0.116 μg/1 μL) in LLV. Symbols represent mean ± SEM. $^{*}p < 0.05$, DMH PBS vs. DMH CNQX (two-way ANOVA, Bonferroni post hoc). The dotted line illustrates the microinjections of PBS or CNQX in DMH and TsTX in LLV.

excitation (Fisher and Coyle, 1991) and supporting the CNS susceptibility to neurological damage triggered by TsTX central action (Guidine et al., 2008a,b, 2009; Lima et al., 1975; Mesquita et al., 2003; Nencioni et al., 2009; Sandoval and Lebrun, 2003). Thus, it is plausible to consider that this hyperexcitability, induced by the excessive glutamate release, could not be counterbalanced by the activation of $GABA_A$ receptors, which could explain, partially, why muscimol injection in DMH did not change the responses evoked by the injection of TsTX. One could suggest that urethane have influenced our results, which is unlikely based on the fact that muscimol injections into the DMH sharply reversed the increase in HR and body temperature caused by prostaglandin E2 injection into the preoptic area in urethane-anaesthetized rats (Zaretskaia et al., 2003), showing that the $GABA_A$ receptors work perfectly in urethane-anaesthetized rats.

In order to study the possibility that the hyperexcitability, induced by the excessive glutamate release, of the DMH is essential to induce the cardiovascular responses evoked by the injection of TsTX, we tested the effect of the blockade of two types ionotropic glutamate receptors (NMDA and AMPA/kainate), in a mixture or separately, within the DMH, in the genesis of cardiocirculatory changes induced by TsTX. Injection of a mixture of NMDA and AMPA/kainate receptor antagonists (AP5 and CNQX, respectively) into the DMH abolished the hypertensive response and decreased the chronotropic changes produced by the TsTX injection. Next, when we blocked the DMH NMDA receptors by injecting AP5 alone, the responses produced by TsTX activation were completely eliminated. Similarly, injecting CNQX (an AMPA/kainate receptor antagonist) resulted in a total reduction of these responses. This mixture concentration, accurately based on literature (de Menezes et al., 2009), was not totally effective in reducing heart rate alterations as isolated antagonists, probably because the dose used in the mixture corresponded to half of dose used in experiments which the antagonists were used separately. In addition, variables as glutamatergic hyperstimulation induced by TsTX and a different sensibility between pressor and chronotropic response could be useful to explain these results. In this sense, higher mixture doses were crucial to promote a total blockade of tachycardia.

It is well known that TsTX inhibits VGSC inactivation and increases excitatory aminoacids (EAA) release (Possani et al., 1999). Importantly, glutamate receptor subtypes are widely present in the hypothalamus, with higher expression in the dorsomedial region (Meeker et al., 1994). Additionally, injections of EAA into the DMH produce increases in HR and blood pressure (De Novellis et al., 1995; Soltis and DiMicco, 1991). Interestingly, glutamate receptor antagonist injections partially/totally blocked the convulsive/neurodegenerative effects produced by the TsTX injection on hippocampus of conscious rats (Nencioni et al., 2003). In fact, NMDA and AMPA/kainate antagonists demonstrate an extensive spectrum anticonvulsant and neuroprotective properties (Nencioni et al., 2003). Thus, based on the results presented in our study and in previous findings, we can suggest that the hypertension and the tachycardia induced by a central injection of TsTX depend on the activation of glutamatergic receptors, both NMDA and AMPA/kainate, of DMH neurons.

Our data suggest that the central circuit recruited by TsTX, whose activation results in an array of physiological and behavioral alterations, depend on the activation of DMH ionotropic glutamatergic receptors. Our data provide new insights on the central mechanisms involved in the development of symptoms in the severe scorpion envenomation syndrome.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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